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13. ABSTRACT (Maximum 200 Words)

This study investigated the association between genetic polymorphisms in hormone producing and metabolizing enzymes and several markers of breast cancer risk among women of different ethnic background. The specific aims were to analyze the relation of breast density and estrogen levels in urine and serum with the presence of variant alleles in *CYP17, COMT, CYP1A1, CYP1A2*, and *CYP1B1*, to describe ethnic differences in urinary excretion levels of estrogen, and to explore the association of breast density with estrogen levels. Mammograms for 328 women were assessed for breast density using a computer-assisted method. The genes were analyzed for polymorphisms using PCR/RFLP methods and estrogens and their metabolites were measured by radioimmunoassay. We found that women carrying the *COMT* and *CYP1A2* variant alleles had lower mammographic densities than women carrying the common alleles. The *CYP1A2* C allele was also significantly associated with lower serum estradiol levels and a lower 2OHE1/16α-OHE1 ratio. Total urinary hormone excretion, androgens, 2-OHE_1, and the 2/16α-OHE_1 ratio were significantly lower in women of Asian ancestry than in Caucasians, but breast density was higher among women of Asian ancestry due to their relatively small breast size. Estrogens and their metabolites measured in the urine of premenopausal women were not associated with mammographic densities. However, contrary to the initial hypothesis, the 2-OHE_1/16α-OHE_1 ratio was directly related with mammographic densities.

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Introduction

A substantial body of evidence indicates that steroid hormones, in particular estrogens, play an important role in the development of breast cancer. Numerous enzymes are involved in the biosynthesis and metabolism of estradiol and estrone, the major circulating estrogens in non-pregnant women. Epidemiologic studies have investigated how a number of genes for enzymes involved in hormone synthesis and metabolic pathways that may be related to breast cancer risk. It is possible that the same genetic variants may also affect mammographic densities, a strong predictor of breast cancer risk. A high percentage of dense parenchyma on mammographic images confers a 4-6 fold risk for breast cancer. The variant alleles may also affect the levels of estrogens and their metabolites in serum and urine.

This study investigated the association between genetic polymorphisms in hormone producing and metabolizing enzymes and several markers of breast cancer risk. The study focused on polymorphisms in five genes (*COMT*, *CYP1A1*, *CYP1A2, CYP1B1,* and *CYP17*) with in women of different ethnic background (Caucasian, Japanese, Native Hawaiian, Filipino, Chinese). This report describes the results for the following detailed objectives:

1. Analyze the relation of breast density with the presence of variant alleles for hormone producing (*CYP17*) and metabolizing enzymes (*COMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1).*
2. Describe ethnic differences in urinary excretion levels of estrogen, its metabolites, and the urinary ratio of 2-hydroxyestrone (2-OHE1)/16α-hydroxyestrone (16α-OHE1) and explore the association of breast density with urinary excretion levels of estrogens, its metabolites, and the 16α-OHE1/2-OHE1 ratio.
3. Examine the association of estrogen levels in urine and serum, as well as the 16α-OHE1/2-OHE1 ratio with polymorphisms in the genes coding for hormone producing (*CYP17*) and hormone metabolizing enzymes *COMT*, *CYP1A1*, *CYP1A2*, and *CYP1B1.*

Body of Report

The results of our research will be presented separately for each objective.

Objective 1. The relation of mammographic densities and genetic polymorphisms

In our published paper "An Investigation of Mammographic Densities and Gene Variants in Healthy Women" (1) (Appendix A), we describe our findings among 328 healthy women of different ethnicities who underwent mammography screening and donated a blood or mouthwash sample for DNA analysis. After digitizing cranio-caudal views of the mammograms, we performed computer-assisted mammographic density assessment. Following DNA extraction, the samples were analyzed for polymorphisms in the *COMT* (Val158Met), *CYP1A1* (Ile462Val), *CYP1B1* (Val432Leu), *CYP1A2 (*1F),* and *CYP17* (T27C) genes using PCR/RFLP methods.

Breast density was lower in Caucasians than in Asians. Caucasian women were less likely to carry the *CYP1A1* variant allele and more likely to carry the variant alleles for *CYP1B1* and *COMT* than women with Asian and Hawaiian ancestry. The low activity *COMT* and *CYP1A2* variant alleles were weakly related to lower percent mammographic density after adjustment for age, ethnicity, body mass index, and reproductive variables (p for gene-dosage effect = 0.08 and 0.05, respectively). These relations were observed in premenopausal women only and were similar in direction and magnitude after stratification by ethnicity. We found no significant associations between breast density and the variant alleles for *CYP1A1*, *CYP1B1,* and *CYP17.* Our data suggest lower mammographic densities for women carrying the *COMT* and *CYP1A2* variant alleles than for women carrying the common alleles, but the direction is in the opposite direction from what is commonly hypothesized from the enzyme function.

Objective 2. Ethnicity, breast density, and urinary excretion of estrogen and metabolites

Our findings "Mammographic Densities and Urinary Hormones in Healthy Women with different ethnic backgrounds" have been published as a chapter in the volume "Hormonal Carcinogenesis IV" edited by Drs. JJ and SA Li (2) (Appendix B). In this analysis, we investigated ethnic differences in urinary hormone levels and their relation with mammographic densities. Because 2-OHE_1 is considered less carcinogenic than 16α-OHE_1, we hypothesized an inverse relation between the 2-OHE_1/16α-OHE_1 ratio and breast densities. Women recruited at mammography clinics completed a questionnaire and donated urine during the luteal phase. Urinary estrone, estradiol, testosterone, and 5α-androstanediol were measured by indirect radioimmunoassays and 16α-OHE_1 and 2-OHE_1 by competitive immunoassays. We assessed mammographic densities with a computer-assisted method and applied multiple linear regression.

The 305 subjects (35-75 years, mean = 47.2 years) reported Caucasian (N = 110), Japanese (N = 86), Hawaiian (N = 35), Chinese (N = 28), and mixed/other ethnicity. BMI, 2-OHE_1, androgens, total hormones, and the 2/16α-OHE_1 ratio were significantly lower in Asians than in Caucasians, but percent densities were 22% higher in Asians. None of the individual hormones was associated with mammographic densities. However, contrary to the initial hypothesis, the 2-OHE_1/16α-OHE_1 ratio was directly related with mammographic densities; the ratio was 25% lower in the lowest density category as compared to the highest category. Our results suggest that the effects of endogenous hormones on breast cancer risk may not be mediated through mammographic densities in adult women.

Objective 3. The association of urinary and serum estrogen with genetic polymorphisms

The final objective was addressed in a manuscript that is currently under review with Cancer Epidemiology, Biomarkers & Prevention. The paper "Association of Genetic Polymorphisms with Serum Estrogens Measured Multiple Times During a 2-Year Period in Premenopausal Women" (Appendix C) explored the association of polymorphisms in genes in the estrogen synthesis and metabolism pathways with serum and urinary levels of estrone and estradiol and with the urinary 2-OHE1/16α-OHE1 ratio. This analysis included 220 women, who were participants in a 2-year randomized soy dietary intervention. Blood specimens were collected in the luteal phase of the menstrual cycle an average of 4.4 times over 2 years. Overnight urinary specimens were collected on the same cycle day, only at baseline. Levels of E1, E2, 2-OHE1, and 16α-OHE1 were measured by enzyme immunoassays. The DNA samples were analyzed by PCR/RFLP for the *COMT* Val158Met, *CYP1A1*2A*, *CYP1A1*2B*, *CYP1A2*1F*, *CYP1B1* Val432Leu, and *CYP17* T27C polymorphisms. We applied mixed models to investigate the relations between genotypes and repeated serum hormone measurements and generalized linear models to assess associations between genotypes and urinary estrogen metabolites.

The *CYP1A2* C allele was significantly associated with lower serum estradiol levels; in CC genotype carriers, serum estradiol levels were 26.3% lower than in homo- and heterozygous common allele carriers combined ($p = 0.01$). *CYP1A2*1F* also affected the urinary 2OHE1/16α-OHE1 ratio; carriers of the variant C allele had a markedly lower ratio than individuals with the AA genotype (1.37 *versus* 1.76; $p = 0.002$). These data suggest that *CYP1A2*1F* is associated with lower circulating levels of estradiol, and that it may be a susceptibility locus for breast cancer.

Key Research Accomplishments

- Breast density as assessed in mammographic images is related to genetic polymorphisms involved in estrogen synthesis and metabolism.
- Women carrying the *COMT* and *CYP1A2* variant alleles were found to have lower mammographic densities for than women carrying the common alleles.
- Total urinary hormone excretion, androgens, 2-OHE_1, and the 2/16α-OHE_1 ratio were significantly lower in women of Asian ancestry than in Caucasians, but percent mammographic densities were higher among women of Asian ancestry due to their relatively small breast size.
- The *CYP1A2* C allele was significantly associated with lower serum estradiol levels.
- Premenopausal carriers of the variant C allele had a markedly lower 2OHE1/16α-OHE1 ratio than CC genotype carriers.
- The fact that *CYP1A2*1F* is associated with lower circulating levels of estradiol and with lower mammographic densities indicates that this polymorphism may be important for breast cancer etiology.
- Estrogens and their metabolites measured in the urine of premenopausal women were not associated with mammographic densities. However, contrary to the initial hypothesis, the 2-OHE_1/16α-OHE_1 ratio was directly related with mammographic densities.
- The effects of endogenous hormones on breast cancer risk may not be directly mediated through mammographic densities in premenopausal women despite the strong relation between mammographic densities and breast cancer risk.

Reportable Outcomes

Peer-reviewed Publications

- Maskarinec G, Lurie G, Williams AE, Le Marchand L. An investigation of mammographic densities and gene variants in healthy women. International Journal of Cancer 2004; 112:683-8.
- Maskarinec G, Williams AE, Rinaldi S, Kaaks R. Mammographic densities and urinary hormones in healthy women with different ethnic backgrounds. In: JJ Li and SA Li (Editors). Hormonal Carcinogenesis IV: Proceedings of the Fourth International Symposium. Springer Science+Business Media, Inc., New York 2005; 277-286.
- Lurie G, Maskarinec G, Stanczyk FS, Kaaks K, Le Marchand L. Polymorphisms Associated with Serum Estrogens Measured Multiple Times during a Two-Year Period in Premenopausal Women. Cancer Epidemiology, Biomarkers, and Prevention. (Under Review).

Published Abstracts (also presented at a conference)

- Maskarinec G, Franke A, Inouye JS, Sepkovic DW, Bradlow HL. Estrogen metabolism in an isoflavone intervention among premenopausal women. Proceedings of the American Association for Cancer Research 2001; 42:311.
- Maskarinec G, LeMarchand L. No relation between CYP17 polymorphism and mammographic density. Cancer Epidemiology, Biomarkers, and Prevention 2002; 11:1222s.
- Lurie G, Maskarinec G, Stanczyk FZ, Le Marchand L. Polymorphisms associated with serum estrogens measured multiple times in premenopausal women. (Poster). Third Annual AACR Annual Conference Frontiers of Cancer Prevention Research. Cancer Epidemiology, Biomarkers & Prevention 2004; 13:1900s.

Presentations

- Maskarinec G, Franke A, Williams AE, Stanczyk FZ. The effects of an isoflavone intervention on the reproductive cycle of premenopausal women (Poster). European Conference on Nutrition & Cancer, Lyon, France, 2001.
- Maskarinec G, LeMarchand L. Genetic Polymorphisms, Estrogens, and Breast Density (Poster). Era of Hope 2002 Breast Cancer Research Program Meeting, Orlando, FL, 9/2002.
- Maskarinec G, LeMarchand L, Kaaks R. An Investigation of Mammographic Densities, Urinary Estrogen Metabolites, and *COMT*, *CYP1A1*, and CYP1B1 Gene Variants in Premenopausal Women (Poster). Molecular and Genetic Epidemiology of Cancer, Kona, HI 1/2003.
- Maskarinec G, Kaaks R. Mammographic densities and urinary hormones in healthy women with different ethnic backgrounds. 4th Annual Symposium on Hormonal Carcinogenesis, Valencia, Spain, 6/2003.

Conclusions

This relatively small study of primarily premenopausal women found evidence that polymorphisms in genes coding for enzymes related to estrogen synthesis and metabolism may affect breast cancer risk. In particular, *CYP1A2*1F* may influence mammographic densities and urinary estrogen excretion. As seen in previous studies by other investigatores, the low activity *COMT* variant allele was also weakly related to lower percent mammographic density. Given the many enzymes that are involved in determining levels of estrogens and their metabolites in serum and urine, studies in large populations need to be performed in order to investigate the combined effect of several polymorphisms. A better understanding of the role of these polymorphisms may elucidate some of the ethnic differences in breast cancer risk and identify susceptible women who can be targeted for cancer screening or prevention programs.

References

(1) Maskarinec G, Lurie G, Williams AE, Le Marchand L. An investigation of mammographic density and gene variants in healthy women. Int J Cancer 2004; 112:683.

(2) Maskarinec G, Williams AE, Rinaldi S, Kaaks R. Mammographic densities and urinary hormones in healthy women with different ethnic backgrounds. In: Li JJ, Li S, Daling JR, editors. Hormonal Carcinogenesis IV: Proceedings of the Fourth International Symposium. New York: Springer Science+Business Media, 2005: 277-286.

Int. J. Cancer: **112,** 683–688 (2004)

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AN INVESTIGATION OF MAMMOGRAPHIC DENSITY AND GENE VARIANTS IN HEALTHY WOMEN

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This cross-sectional study examined if polymorphisms in genes that code for enzymes involved in the production and metabolism of estrogens are associated with mammographic density, a strong predictor of breast cancer risk. The study included 328 healthy women of different ethnicities who underwent mammographic screening and donated a blood or mouthwash sample for DNA analysis. After digitizing craniocaudal views of the mammograms, we performed computer-assisted mammographic density assessment. Following DNA extraction, samples were analyzed for polymorphisms in the *COMT* (Val158Met), *CYP1A1* (Ile^{462}Val), *CYP1B1* (Val^{432}Leu), *CYP1A2* (*1F) and *CYP17* (T27C) genes using PCR-RFLP. Breast density was lower in Caucasians than in Asians. Caucasian women were less likely to carry the *CYP1A1* variant allele and more likely to carry the variant alleles for *CYP1B1* and *COMT* than women with Asian or Hawaiian ancestry. The low-activity *COMT* and *CYP1A2* variant alleles were weakly related to lower percent mammographic density after adjustment for age, ethnicity, body mass index and reproductive variables (p for gene-dosage effect = 0.08 and 0.05, respectively). These relations were observed in premenopausal women only and were similar in direction and magnitude after stratification by ethnicity. We found no significant associations between breast density and the variant alleles for *CYP1A1, CYP1B1* and *CYP17*. Our data suggest lower mammographic density for women carrying the *COMT* and *CYP1A2* variant alleles than for women carrying the common alleles, though this is the opposite of what is commonly hypothesized from the enzyme function.

Key words: *mammographic density; gene variant; breast cancer*

A substantial body of evidence indicates that steroid hormones, in particular estrogens, play an important role in the development of breast cancer.[1–4] Numerous enzymes are involved in the biosynthesis and metabolism of estradiol and estrone, the major circulating estrogens in nonpregnant women. Epidemiologic studies have investigated how a number of genes for enzymes involved in hormone synthesis and metabolic pathways may be related to breast cancer risk. Previous studies have focused on members of the CYP family and on COMT.[5–7] The enzyme CYP17 plays a role in the formation of precursor androgen steroids that can be transformed to active estrogens.[8] Whereas the C2 hydroxylation of estrogens is primarily mediated by the enzymes CYP1A1 and CYP1A2, CYP1B1 appears to be more active in C4 hydroxylation.[9] COMT inactivates and detoxifies the catechol C2- and C4-hydroxy estrogens to their respective methoxy metabolites.[10] The 4-hydroxy estrogen metabolites are considered more carcinogenic than the 2-hydroxy metabolites, which are thought to inhibit breast cell proliferation.[7,11] Specific polymorphisms in these genes may lead to a change in enzyme activity and, thus, may modulate endogenous hormone levels and breast cancer risk (Table I). The functionality of enzymatic products from variant genes has been investigated *in vitro* for some polymorphisms.[7] So far, case-control studies exploring associations between these gene variants and breast cancer risk have presented conflicting results.[5–7,12]

It is possible that the same genetic variants may also affect mammographic density, the radiographic patterns describing the relative distribution of fat, connective and epithelial tissue in the female breast. A high percentage of dense parenchyma on mammographic images, which appears to confer a 4- to 6-fold increased risk for breast cancer,[13] has a genetic component, based on the significant correlations with breast density observed between sisters in a cohort study[14] and the 2-fold higher correlation between monozygotic compared to dizygotic twins.[15] A role of steroid hormones in mammographic density is supported by observations of an increase in density after HRT[16,17] and a decrease after suppression of ovarian function through a gonadotropin-releasing hormone agonist[18] or after tamoxifen treatment.[19] However, the only study to investigate circulating sex hormones and breast density[20] found that estrogen and progesterone levels were unrelated to percent density. Because some metabolites of endogenous estrogens are more estrogenic than others, genetically determined differences in biosynthesis and metabolic pathways of estrogens may affect breast cancer risk as reflected in mammographic density. We focused on the association of polymorphisms in 5 genes (*COMT, CYP1A1, CYP1A2, CYP1B1* and *CYP17*) with mammographic density in women of different ethnic backgrounds. With the exception of *CYP1A2*, the relation of breast density with these polymorphisms has been investigated in previous studies and remains undefined.[21–23] Based on the hypothesized effects of the variant alleles on enzyme function (Table I), a higher breast cancer risk was proposed for polymorphisms in 4 genes, with the exception of *CYP1A1*.

MATERIAL AND METHODS

Study design and population

We included 328 women who were participants in 3 previous studies that involved mammographic density: 98 from a cross-sectional study,[24] 8 from an isoflavone trial[25] and 222 from a soy intervention trial.[26] We obtained approval from the Committee on Human Studies at the University of Hawaii and from the institutional review boards of the participating hospitals for the original studies and for the added genetic component. All subjects gave written informed consent to participate in the research projects. Women from all 3 studies were recruited in mammography screening clinics in Honolulu through mailed invitations that asked interested women to contact our center. Eligible women had to have a normal mammogram at recruitment and no previous history of cancer or breast surgery. For the intervention studies, baseline mammograms were used for analysis. Women with any level of

Abbreviations: BMI, body mass index; CI, confidence interval; COMT, catchol-*O*-methyltransferase; CYP, cytochrome P-450; HRT, hormone replacement therapy; ICC, intraclass correlation coefficient; RFLP, restriction fragment-length polymorphism.

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TABLE I – HYPOTHESIZED AND OBSERVED RELATIONS OF VARIANT ALLELES WITH BREAST DENSITY

Gene	Encoded product	Polymorphism	Function of enzyme/hypothesized effect of variant allele	Expected effect on breast cancer risk	Observed effect on breast density (present study)
COMT	Catechol-*O*-methyltransferase	G⇒A (Val158Met)	Inactivates catechol (2- and 4-OH) estrogen metabolites/reduced activity, higher levels of catechol metabolites	⇑	⇓
CYP1A1	Cytochrome P-450 1A1	A⇒G (Ile462Val)	Catalyzes hydroxylation of 17β-E_2 at C-2 and to lesser degree at C-4/ increased activity, more 2-OH and fewer potentially carcinogenic 4-OH estrogen metabolites	⇓	⇑
CYP1A2	Cytochrome P-450 1A2	C⇒A (A-164C)	Catalyzes hydroxylation of 17β-E_2 at C-2 and to lesser degree at C-4/ decreased activity, fewer 2-OH and more potentially carcinogenic 4-OH estrogen metabolites	⇑	⇓
CYP1B1	Cytochrome P-450 1B1	G⇒C (Val432Leu)	Produces primarily potentially carcinogenic 4-OH estrogen metabolites/increased activity, more 4-OH metabolites	⇑	⇓
CYP17	Cytochrome P-450 c17α	T27C	Mediates steroid 17α-hydroxylase and 17,20-lyase activities/increased estrogen production	⇑	⇑

breast density were eligible to participate. Subjects for the intervention were also free of serious medical conditions, reported regular menstrual periods and an intact uterus and ovaries, did not use oral contraceptives or any hormone preparations at the time of enrolment and within the past 3 months and had no intention of becoming pregnant. Due to the nature of the nutritional intervention, only women who reported a dietary consumption of <7 servings of soy food/week were eligible. All subjects completed a validated food-frequency questionnaire,[27] especially designed for our multiethnic population, and detailed questions on reproductive, medical and family histories as well as body height and weight. Subjects donated a blood (90%) or mouthwash (10%) sample for genetic analysis; these were stored in a freezer at –70°C until analysis.

Genetic analysis

DNA was purified from whole blood using Qiagen (Valencia, CA) Midi Kits. DNA samples were analyzed by PCR-RFLP using published methods for the *COMT* Val158Met, *CYP1A1* Ile462Val, *CYP1A2*1F* (A-164C), *CYP1B1* (Val432Leu) and *CYP17* (T27C) polymorphisms.[28–32]

Mammographic density assessment

Cranio-caudal views of the mammogram were obtained from the mammography clinics after radiologic evaluation had been completed and had ruled out any malignancy. Films were scanned using a Kodak (Rochester, NY) LS-85 X-ray digitizer with a pixel size of 260 μm (equal to a resolution of 98 pixels/inch). One of the authors (G.M.) performed computer-assisted mammographic density assessment using a program developed in Canada.[33] The reader first chooses a threshold gray value that defines the outline of the breast and then selects the best threshold to identify the regions that represent mammographic density. The pixel count corresponding to the dense area is determined by the computer, as is the total area within the outline of the breast. Percent density was calculated as the ratio of the dense area to the total area of the breast multiplied by 100. A random sample of 58 mammograms was read blindly in duplicate. ICCs[34] were 0.95 (95% CI 0.92–0.97) for the size of the dense areas and 0.98 (95% CI 0.97–0.99) for the total breast area, resulting in an ICC of 0.97 for percent density (95% CI 0.95–0.98).

Statistical analysis

The SAS statistical software package, version 8.2 (SAS Institute, Cary, NC), was used for data management and statistical analysis. BMI was calculated as the ratio of weight (kg) divided by the square of height (m). In the questionnaire, subjects marked all ethnic backgrounds that applied to themselves and to their parents. We assigned summary categories according to the following rules. Women with any Hawaiian background were classified as *Native Hawaiian.* A woman was classified as *Caucasian* if both parents had some Caucasian ancestry and shared no other ethnic background. Subjects who reported no more than 3 ethnic backgrounds were classified as *Chinese, Japanese* or *Filipino* if both parents were of Asian ancestry or if the mother was of the respective ethnic background and the parents shared no other ethnic background. Finally, the *Other* category included Pacific Islanders, African Americans, Latinas and women with mixed ethnic backgrounds that did not fit one of the above groups. Because of the small sample size, subjects were combined into 3 major ethnic categories for statistical analysis: Caucasian, Asian (Chinese, Filipino, Japanese), Mixed/Other (Hawaiian, mixed, other). Tukey's multiple comparison test[35] indicated that there was no statistically significant difference in breast density between the 90 Japanese, 30 Chinese and 13 Filipino women before and after adjustment for covariates. After computing mean percent densities for women by genotype, we applied generalized linear models (PROC GLM) to test for associations between genotype and percent density and to calculate least-squares means by genotype, while adjusting for potential confounders (age, BMI, ethnicity, reproductive variables).[35] Age and BMI were treated as continuous variables, whereas genotype, ethnicity, age at first live birth, parity, menopausal status and HRT use were modeled with indicator variables. Gene-dosage effects were modeled by assigning a value of 0, 1 or 2 to a genotype trend variable according to the subject's number of variant alleles as described previously.[36,37]

RESULTS

The mean age of the 328 subjects was 46.8 ± 7.8 years (range 34–85) (Table II). The great majority of women were premenopausal (81.4%) and born in the United States (91.7%). Of the 133 women classified as Asian (90 Japanese, 30 Chinese, 13 Filipino), only 20 reported more than one ethnicity, but this was a non-Asian background for only 14 women. The Mixed/Other category (n = 77) included 37 women with native Hawaiian ancestry. Average BMI was 25.0 ± 5.2 kg/m^2 with significant differences among ethnic groups (p = 0.0002). BMI was highest in Hawaiians (27.9 kg/m^2), intermediate in Caucasians and Filipinas (25.7 and 25.1 kg/m,^{2} respectively) and lowest in Chinese and Japanese

TABLE II – MEAN PERCENT MAMMOGRAPHIC DENSITY BY STUDY CHARACTERISTICS

Characteristic	Number (%)	Percent mammographic density	
		Mean	Adjusted mean[1]
Ethnic category			
Caucasian	118 (36)	35.2	36.6
Asian	133 (41)	44.3	43.0
Mixed/other	77 (23)	40.6	41.8
		$p^2 = 0.007$	$p = 0.03$
Menopausal status			
Premenopausal	268 (82)	42.3	43.1
Postmenopausal	60 (18)	30.5	43.4
		$p = 0.0003$	$p = 0.70$
Hormone replacement			
Yes	29 (52)	26.1	35.9
No	31 (48)	34.5	29.5
		$p = 0.13$	$p = 0.25$
Age (years)			
<40	35 (11)	37.5	41.7
40–49	220 (67)	44.4	47.0
50–59	45 (14)	28.2	25.9
≥60	28 (8)	29.3	22.6
		$p = 0.0006$	$p < 0.0001$
BMI (kg/m^2)			
<18.5	18 (6)	48.1	53.2
18.5–24.9	184 (56)	46.9	48.8
25–29.9	76 (23)	33.4	34.7
≥30	50 (15)	22.8	23.1
		$p < 0.0001$	$p < 0.0001$
Age at menarche (years)			
<13	202 (62)	39.2	41.9
≥13	126 (38)	41.8	41.4
		$p = 0.32$	$p = 0.82$
Parity (number of children)			
0–2	234 (73)	42.4	43.2
3+	88 (27)	34.2	37.8
		$p = 0.004$	$p = 0.06$
Age at first live birth (years)			
<30	166 (51)	37.3	41.2
≥30	162 (49)	43.1	42.2
		$p = 0.02$	$p = 0.71$

[1]Adjusted for all other variables in the table using least-square means in a general linear model.
[2]p values are for the global test for differences between groups.

(22.8 and 23.9 kg/m,[2] respectively). There were no significant differences among ethnic groups by menopausal status, parity, age at first live birth and HRT use.

The average percent mammographic density in the study population was 40.2 ± 23.0% (Table II). Mammographic density showed significant inverse associations with age and BMI. Breast density was lowest in Caucasians (35.2 ± 22.9%) and highest in women of Asian ancestry (44.3 ± 22.8%). These differences remained significant after adjustment for age, BMI, menopausal status, parity, age at first live birth, menopausal status and HRT use. Postmenopausal women had 11.9% lower densities than premenopausal women. Among the small number of women currently using HRT ($n = 29$), breast density was unexpectedly lower than among women who were not currently using HRT (26.1 *vs.* 34.5%); but this difference was not statistically significant ($p = 0.13$) and was reversed after adjusting for covariates. Due to the inclusion criteria for the 2 intervention studies, only 7 premenopausal women were taking oral contraceptives. Their breast density was not significantly different from that of the rest of the population. Women who had at least 3 children had 8.2% less density than women with 2 or fewer children. Age at first live birth younger than 30 years was associated with 6.2% lower percent density. Age at menarche was not related to mammographic density.

The distributions of the studied genotypes were all consistent with Hardy-Weinberg equilibrium within ethnic groups. We found significantly different genotype distributions by ethnicity for *COMT*, *CYP1A1* and *CYP1B1* ($p < 0.0001$). Caucasian women had a significantly higher prevalence of the variant alleles for *COMT* and *CYP1B1* (58% and 46%, respectively) than women of Asian (30% and 20%) and Mixed/Other (27% and 35%) ancestry. However, the prevalence of both variant alleles for *CYP1A1* was significantly lower in Caucasians (8%) than in women of Asian (29%) and Mixed/Other (21%) ancestry. We observed no significant ethnic differences for the *CYP1A2* and *CYP17* genotypes ($p = 0.07$ and 0.27, respectively). BMI was not associated with the genotypes *COMT* ($p = 0.83$), *CYP1A2* ($p = 0.24$) and *CYP17* ($p = 0.33$) but showed an inverse relation with *CYP1A1* ($p = 0.04$) and a positive association with *CYP1B1* ($p = 0.02$).

The *COMT* ^{158}Met allele was inversely related to percent density with a gene-dosage effect ($p = 0.01$) (Table III). Homozygous carriers of the *COMT* variant allele had 8.9% lower percent mammographic densities than carriers of 2 common *COMT* alleles. Adjustment for confounders reduced this difference to 6.3%, and the test for a gene-dosage effect was of borderline significance ($p = 0.08$). For premenopausal women, the difference in density between extreme genotypes was 9.3% and the gene-dosage effect was statistically significant ($p = 0.03$). Carriers of the *CYP1A1* variant allele had higher mammographic densities than common allele homozygotes. The difference in mean percent density was 14.5% (p for gene dosage = 0.01), but after adjustment for confounders, it was diminished to 6.0% (p for gene dosage = 0.39). Women with variant alleles for *CYP1A2* had lower percent densities than subjects with the corresponding common alleles; after adjustment for confounders, the difference in mean density between the 2 groups was 6.8% and the test for gene-dosage effect was significant ($p = 0.049$). This difference was greater in premenopausal women (11%, p for gene dosage = 0.048). However,

TABLE III – MEAN MAMMOGRAPHIC PERCENT DENSITY BY GENOTYPE

Gene (polymorphism)	Genotype	All women					Premenopausal only		
		Number	Mean percent density	p^1	Adjusted mean #	p^1	Number	Adjusted mean[2]	p^1
COMT (Val158Met)	Val/Val	129	43.7	0.01	42.0	0.08	112	44.9	0.03
	Val/Met	140	39.0		40.7		109	42.8	
	Met/Met	58	34.8		35.7		46	35.6	
CYP1A1 (Ile462Val)	Ile/Ile	216	38.3	0.01	40.0	0.39	179	42.8	0.75
	Ile/Val	95	42.0		40.4		73	40.6	
	Val/Val	16	52.8		46.0		15	49.3	
*CYP1A2*1F* (A-164C)	A/A	173	41.5	0.25	42.4	0.049	151	44.2	0.048
	A/C	131	38.8		38.7		100	41.7	
	C/C	23	37.3		35.6		16	33.2	
CYP1B1 (Val432Leu)	Val/Val	143	43.5	0.04	41.7	0.89	121	44.2	0.60
	Val/Leu	152	37.5		38.4		121	40.3	
	Leu/Leu	32	37.5		44.8		25	45.8	
CYP17 (T27C)	CC	113	38.1	0.30	38.6	0.17	93	41.1	0.49
	CT	158	41.2		41.1		130	43.2	
	TT	57	41.4		42.8		45	43.6	

[1]*p* values for gene-dosage term assigned 1, 2 and 3 for 0, 1 and 2 variant alleles, respectively.–[2]Adjusted for age, ethnicity, BMI, parity, age at first live birth, menopausal status and HRT use.

the statistically significant association of breast density with *CYP1B1* (p for gene dosage = 0.04) completely disappeared in the adjusted model (p for gene dosage = 0.89). The presence of the variant allele for *CYP17* showed little relation with breast density before and after adjustment (p for gene dosage = 0.30 and 0.17, respectively).

After stratification by ethnic category, homozygous carriers of the *COMT* variant allele had lower percent densities than carriers of the common allele in all 3 ethnic categories, but the respective differences by genotype (5.6%, 3.5% and 6.2%) were somewhat smaller than in the entire study population (6.3%) and not statistically significant (Table IV). For *CYP1A2*, a nonsignificant pattern of lower breast density in women with variant alleles was also observed in all 3 groups (6.6%, 2.6% and 12.2%, respectively). As for the overall population, the results by ethnic group did not suggest any associations for carriers of the *CYP1A1, CYP1B1* or *CYP17* variant alleles. A separate analysis of the small number of postmenopausal women (data not shown) did not indicate an association of the *COMT* or *CYP1A2* genotype with breast density in that subgroup.

DISCUSSION

In our study of primarily premenopausal women, the presence of either the *COMT* 158Met or the *CYP1A2*1F* variant allele was related to lower mammographic density. After adjustment for confounders, these associations were weakened, but they were statistically significant when the sample was limited to premenopausal women. The fact that the associations for both genotypes showed some consistency across ethnic groups argues against residual confounding by ethnicity. After adjustment for confounders, mammographic density did not show any relation with the presence of variant alleles for the *CYP1A1, CYP1B1* and *CYP17* genes, overall or when stratified by ethnic group. In comparison to

TABLE IV – MEAN MAMMOGRAPHIC PERCENT DENSITY BY GENOTYPE AND ETHNIC CATEGORY

Gene (polymorphism)	Genotype	Percent density[1]					
		Caucasian		Asian		Mixed/Other	
		Number	Mean	Number	Mean	Number	Mean
COMT (Val158Met)	Val/Val	20	35.9	66	46.6	43	41.3
	Val/Met	60	38.8	53	41.9	27	41.2
	Met/Met	38	30.3	13	43.1	7	35.1
	p^2 =		0.17		0.27		0.64
CYP1A1 (Ile462Val)	Ile/Ile	100	36.0	68	43.3	48	40.7
	Ile/Val	17	32.3	52	43.1	26	41.9
	Val/Val	1	40.5	12	55.6	3	30.9
	p =		0.58		0.17		0.76
*CYP1A2*1F* (A-164C)	A/A	64	36.6	66	47.4	43	42.9
	A/C	48	34.7	58	41.0	25	40.8
	C/C	6	30.0	8	44.8	9	30.7
	p =		0.42		0.18		0.16
CYP1B1 (Val432Leu)	Val/Val	31	45.1	84	43.6	28	38.3
	Val/Leu	65	29.7	43	45.2	44	41.3
	Leu/Leu	22	38.9	5	51.0	5	48.2
	p =		0.10		0.43		0.37
CYP17 (T27C)	CC	47	34.7	38	42.7	28	36.1
	CT	59	35.6	65	45.7	34	42.5
	TT	12	38.2	30	44.4	15	45.4
	p =		0.61		0.69		0.15

[1]Adjusted for age, ethnicity, BMI, parity, age at first live birth, menopausal status and HRT use.–[2]*p* values for gene-dosage term assigned 1, 2 and 3 for 0, 1 and 2 variant alleles, respectively.

the hypothesized effects of variant alleles on breast cancer risk (Table I), the direction of the observed association with mammographic density was contrary to expectation for *COMT* and *CYP1A2*. Since there is little evidence at this time that circulating estrogens or their metabolites are directly associated with mammographic density,[20] the previously hypothesized mechanism for a relation between estrogens, the genetic polymorphisms studied here and breast density may have to be reconsidered. Estrogens may play a less important role in determining mammographic density than progesterone or other hormones. In a clinical trial, mammographic density increased 3–5% in women taking any treatment containing a progestin, whereas women in the estrogen-only group experienced a nonsignificant increase of 1.3%.[17] We found that the prevalence of variant alleles for *COMT, CYP1B1* and *CYP1A1* differed significantly by ethnicity, with a higher prevalence of the *COMT* and *CYP1B1* variant alleles and a lower prevalence of the variant alleles for *CYP1A1* in Caucasians than in women of all other ethnicities. These distributions by ethnicity agree with published reports.[23,36]

Three other studies have investigated the relation of breast density with polymorphisms in the *COMT* gene.[21–23] Similar to our results, a Canadian cross-sectional investigation[23] described 14.2% lower mammographic density for premenopausal women who were homozygous for the *COMT* 158Met variant allele compared to women with 2 common alleles but found no association among postmenopausal women. Interestingly, it appeared that this relation was at least in part mediated by body size and serum levels of growth hormone, IGF-I, IGF binding protein-3, follicle-stimulating hormone and progesterone. In a study of Caucasian and African-American breast cancer cases in Los Angeles, current HRT users who were carriers of the low-activity *COMT* 158Met allele had greater breast density[21] but no association was observed for the entire study population. Finally, an analysis of the Nurses' Health Study[22] found no statistically significant association between the *COMT* genotype and breast density but described mean percent densities for the homozygous common and homozygous variant genotypes among premenopausal women that were similar to those in our study (43.1% and 33.9%, respectively). Also consistent with our results and the Canadian study,[23] no association was observed in postmenopausal women. The *CYP17* polymorphism was not related to breast density in either of the 2 published reports.[21,22] One study reported null findings for *CYP1A1* and *CYP1B1*,[21] but we are not aware of any report investigating breast density and polymorphisms of *CYP1A2*. Investigations of breast cancer risk with the genetic polymorphisms studied here have yielded contrasting results.[5–7,12] As summarized,[7] the majority of studies on *COMT, CYP1A1* and *CYP17* have found no significant associations in the entire study population. In several reports, a particular subgroup, *e.g.*, premenopausal women, smokers, advanced cases or women with later age at menarche, experienced a reduced or increased risk related to the variant allele.

The main limitation of our study was the small sample size, which provided inadequate statistical power to detect possible associations in subgroups. Although inclusion of different ethnic groups has the advantage of increasing the variance of the outcome variable, *i.e.*, mammographic density, it limits the interpretation of results. As reviewed by Hirschhorn *et al.*,[38] population stratification may lead to spurious associations in genetic studies. This bias derives from the fact that a genetic factor that differs between ethnic groups may falsely appear to be related to the outcome. Unmeasured confounders and other functionally relevant polymorphisms specific to some ethnic groups may thus bias results. However, as mentioned above, our findings appear consistent across ethnic groups, suggesting that population stratification may not have been an issue. Also, genotype alone does not measure the complete phenotypic effect[7,12] because enzyme activity may be induced or inhibited by environmental factors. Furthermore, since the majority of women were selected according to strict inclusion criteria for 2 nutritional interventions, they may not have been representative of the general population and the results may not be generalizable. However, it does not appear likely that selection factors could modify the relationship between genotype and mammographic density.

In conclusion, we investigated the distribution of genetic variants suspected to modify the biosynthesis and metabolism of endogenous estrogens and the relation of these polymorphisms with mammographic density among healthy women with different ethnic backgrounds. Because of their high prevalence, these genes have the potential to play a significant role in breast cancer etiology even if there is only a low individual risk associated with them. If the search for susceptibility genes were successful, it would be possible to identify high-risk women for prevention efforts by developing multigenic models of breast cancer susceptibility.[39] Although our data suggest lower mammographic density for women carrying variant alleles for *COMT* and *CYP1A2*, much larger populations will be required to reach this goal and to shed light on the mechanisms underlying these associations.

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HORMONAL CARCINOGENESIS IV

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Mammographic Densities and Urinary Hormones in Healthy Women with Different Ethnic Backgrounds

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Summary

An association between sex steroids and mammographic density (MD), a predictor of breast cancer (BC) risk, is supported by findings of increased densities after hormone therapy and reduced densities after tamoxifen treatment. Herein, ethnic differences in urinary hormone levels and their relation to MDs were investigated. Because 2-hydroxyestrone (2-OH-E_1) is considered less carcinogenic than 16α-hydroxyestrone (16α-OH-E_1), we hypothesized an inverse relation between the 2-OH-E_1/16α-OH-E_1 (2/16) ratio and breast densities. Women recruited completed a questionnaire and donated urine during the luteal phase. Urinary estrone (E_1), estradiol (E_2), testosterone (T), and 5α-androstane-3α, 17β-diol (ADIOL) were measured by indirect radioimmunoassay (RIA) and 16α- and 2-OH-E_1 by competitive immunoassays. MDs were assessed with a computer-assisted method and applied multiple linear regressions. The total number of subjects was 305 (35-75 years, $\bar{x}$ = 47.2 years). Their ethnic distribution was Caucasian (110), Japanese (86), Hawaiian (35), Chinese (28), and mixed/other ethnicity (46). The data indicate that the body mass index (BMI), 2-OH-E_1, androgens (A), total hormones, and the 2/16 ratio were significantly lower in Asians than in Caucasians, but the % MDs were 22% higher in Asians. None of the individual hormones was associated with MDs. However, contrary to the initial hypothesis, the 2/16 ratio was directly related to MDs. The ratio was 25% lower in the lowest MD category as compared to the highest category. The data suggest that the effects of endogenous hormones on BC risk may not be mediated through MDs in adult women.

Introduction

MD patterns refer to the distribution of fat, connective, and epithelial tissue in the breast, and are strong predictors of BC risk. A high percentage of dense

parenchyma on mammographic images confers a 4.0- to 6.0-fold risk to develop BC (1). Endogenous estrogens (Es) and perhaps As are important in the etiology of BC (2-4). An association of MDs and hormone levels is supported by several observations. Hormone replacement therapy (HRT) increases MD (5). In the other hand, tamoxifen treatment improves MDs (6) by suppressing ovarian function through a gonadotropin-releasing hormone agonist (7). Moreover, MDs returned to baseline after tamoxifen was discontinued (8). However, two cross-sectional investigations detected no strong relation between MDs and serum E and progesterone (P) levels (9, 10).

Because some metabolites of endogenous Es may have more estrogenic effects than others, we hypothesized that differences in E metabolic pathways may be related to MDs. The metabolism of E_2 follows two major competing pathways, C2- and C16α-OH-lation, and a minor C4-OH-lation (11-13). It has been proposed that women who metabolize a larger proportion of their endogenous Es through 16α-OH-lation are at greater BC risk because 16αOH-E_1 has genotoxic effects, damages DNA, and enhances breast cell growth, whereas 2-OH-E_1 inhibits breast cell proliferation. However, before transformation into methoxy compounds by the enzyme catechol methyl transferase (COMT), 2-OH compounds have some estrogenic and growth promoting effects (13). The evidence on the association of the 2/16 ratio with BC is inconsistent (14-18), and a previous study on MDs reported results contrary to the original hypothesis (19). Women in the highest tertile of the 2/16 ratio were 6.0 times more likely to have a high risk mammographic pattern. In disagreement with the hypothesis that women from ethnic groups with lower BC risk have a higher 2/16 ratio, a comparison between Finnish and Asian women (20) reported a higher ratio in Finnish women. Herein, ethnic differences in urinary E, A, and E metabolite levels and their relation with MD were investigated.

Materials and Methods

Study Design and Population. This study included 305 women, participants of three studies involving MD, 97 were from a cross-sectional study (21), 7 from an isoflavone intervention study (22), and 201 from a soy intervention study (23). For the latter two studies, baseline mammograms and urine samples were used for the analysis. After the approval of the Committee on Human Studies at the University of Hawaii, all subjects provided informed consent. Women for all three studies were recruited at mammography clinics in Honolulu. Eligible women had no previous history of BC and required a normal mammogram at baseline. The subjects for the intervention studies were free of serious medical conditions, had regular menstrual periods, intact uterus and ovaries, were not on oral contraceptives or other hormones, and had no intention of becoming pregnant within a year. Due to the nature of the nutritional intervention, only women who reported a dietary consumption of ≤ 7 servings of soy food/week were eligible. All subjects completed

a validated food frequency questionnaire (24), which included questions on reproductive, medical, and anthropometric factors. In the cross-sectional study, self-reported information was used to classify women's menopausal status and hormone use (HRT). The majority of subjects (n = 208) donated an overnight urine specimen, while those of the cross-sectional study (n = 97) only a spot urine sample.

Mammogram Density Assessment. Cranio-caudal views of the mammogram were obtained from the clinics after complete radiologic evaluation and ruled out any malignancy. The films were scanned into a PC using a Kodak LS-85 X-ray digitizer with a pixel size of 260 μm (resolution = 98 pixels/inch). One of the authors (GM) performed computer-assisted MD assessment using a Canadian method (25). The reader chooses a threshold value that defines the outline of the breast, and then selects the best threshold to identify the regions that represent MDs. The pixel count corresponding to the dense area is determined by the computer, as is the total area within the outline of the breast. Percent MD was calculated as the ratio of the dense area to the total area of the breast multiplied by 100. A random sample of 58 mammograms was read in duplicate. The intra-class correlation coefficients (26) were 0.95 (95% CI, 0.92-0.97), for the size of the dense areas, and 0.98 (95% CI, 0.97-0.99) for the total breast area, and of 0.97% density (95% CI, 0.95-0.98).

Urinary Hormone Analysis. Urine concentration of E_1, E_2, T, and ADIOL was performed with slight modifications previously published (27). Briefly, 1.0-ml urine samples were hydrolyzed, purified by solid phase extraction, and HPLC. Hormone concentrations were measured by RIA on the dried extracts. All measurements were done in duplicate, including hydrolysis, solid phase extraction, HPLC, and RIA. For quality control, two control samples containing known amounts of steroids were included for all the analytical steps in each sample batch. The detection limits were: 0.02 ng/ml for E_1 and E_2, 0.08 ng/ml for ADIOL, and 0.02 ng/ml for T. Intra- and inter-batch coefficients of variations were 4.7 & 16%, respectively, for E_1 (at 3.3 ng/ml), 1.7% & 14% for E_2 (at 0.32 ng/ml), 4.7% & 14% for T (at 3.3 ng/ml), and 7.0% & 11% for ADIOL (at 15.5 ng/ml). 2-OH- and 16α-OH-E_1 were measured by solid-phase enzyme immunoassays after enzymatic hydrolysis with Helix Pomatia (Estramet, Immunacare Co., Bethlehem, USA). Mean intra- and inter-batch coefficients of variations were 10 & 15%, respectively, for both analytes.

Statistical Analysis. The SAS statistical software package version 8.2 (SAS Institute Inc., Cary, NC) was used for data management and statistical analyses. In the questionnaire, subjects marked all ethnic backgrounds that applied to themselves and to their parents. Summary categories were assigned according to the following rules: A woman was classified as Caucasian if both parents had some Caucasian ancestry and shared no other ethnic background. Subjects with no more than three ethnic backgrounds were classified as Chinese, Japanese, or Filipino, if both parents were of the same ethnicity or if the mother was of the respective ethnic background

and the parents shared no other ethnic background. Because of the similarity in percent MDs, the 86 Japanese, 28 Chinese, and 9 Filipino women were combined into one Asian category. In agreement with rules applied in the State of Hawaii (28), women with any Hawaiian background were classified as Native Hawaiian. Because of their mixed ancestries, the Native Hawaiian women (n = 35) were included into the other category containing Pacific Islanders, African-Americans, Latinas (n = 24), and women with mixed ethnic backgrounds that did not fit any of the above categories (n = 13).

Body Mass Index. BMI was calculated as the ratio of weight in kg divided by the square of the height in m. Non-normally distributed variables were transformed using their natural logarithm. Percent MD was classified into five categories: <10%, 10 to 24.9%, 25 to 49.9%, 50 to 74.9%, and ≥75%. To explore associations between MD and urinary hormone measurements, we computed Spearman correlation coefficients (r_s) and included potential confounders (29). Then, we applied analysis of variance to test for associations between ethnicity, hormone levels, and mammographic characteristics with adjustment for confounding variables (30). In addition, we computed least-squares means for the urinary hormone levels by category of percent MD using the proc glm procedure in the SAS software package (30). Finally, we performed trend tests to investigate a possible relation between the different hormones and percent MD. We regressed the mean level of the hormones onto the mean MD of each of the five MD categories.

Results

Characteristics of study population. Most of the 305 women were premenopausal; only 25% were postmenopausal (Table 1). The mean age was 47.2 years. Caucasians were slightly younger than Asian women. The BMI was lowest among Asian, intermediate in Caucasians, and highest in the mixed/other category (p = 0.0004). Percent MD differed significantly by ethnicity (p = 0.003), even after adjustment for age, menopausal status, and BMI (p = 0.03). Percent MD was highest among Asians and lowest among Caucasians, 45.2% vs. 34.9%. Of the 305 women, 35, 48, 108, 87, and 27 belonged to the five MD categories. Close to 40% of Caucasians were in the two lowest MD categories, but only 20% of Asians were classified that way. MD density was significantly higher for pre than for postmenopausal women (44.2 ± 23.0% vs. 28.1 ± 19.2%). The MD difference between Caucasians and Asians was greater after menopause than before (16% vs. 9%).

Excretion of all combined hormones was lowest among Asians and similar in the two other groups, but this difference was not statistically significant (p = 0.09). The difference in hormone levels was primarily due to 2-OH-E_1, ADIOL, and T, which differed significantly by ethnicity, even after stratification for menopausal status or HRT use. While A levels did not vary by HRT use, E levels were approximately 2.0-fold higher among postmenopausal women on HRT than among non-users. After stratification by menopause and HRT use, A levels remained lower

among Asian than Caucasian women in each subgroup. Levels of 16α-OH-E_1, E_1, and E_2 were similar in the three ethnic groups. The 2/16 ratio was approximately 25% lower in Asian women than in the other two groups ($p = 0.04$). The ethnic difference in the 2/16 ratio was greater before (1.56 vs. 2.11) than after menopause (1.55 vs. 1.72) for Asian and Caucasian women, respectively. The 2/16 ratio did not differ by HRT use ($p = 0.57$).

Table 1. Characteristics of the Study Population.

Variable	Asian	Mixed/ Others	Caucasian	All		p-value[1]
				Mean	Std	
Number	123	72	110	305		--
Menopausal (%)	28	18	24	25		0.32
Age (years)	48.50	45.40	46.80	47.20	8.00	0.03
BMI (kg/m^2)	23.40	26.40	25.70	24.90	5.10	0.0004
Percent MD	45.16	39.90	34.92	40.23	23.13	0.003
E_1 (ng/ml)	11.82	14.27	11.90	12.43	19.78	0.68
E_2 (ng/ml)	3.90	4.85	3.84	4.11	5.03	0.43
2-OH-E_1 (ng/ml)	16.39	19.54	20.99	18.80	14.07	0.04
16α-OH-E_1 (ng/ml)	11.60	12.52	11.08	11.63	8.45	0.52
2/16 ratio	1.56	1.81	2.02	1.79	1.00	0.0006
T (ng/ml)	2.83	4.06	4.16	3.60	2.94	0.0007
ADIOL (ng/ml)	19.94	25.78	21.82	22.00	17.26	0.07
All hormones (ng/ml)	67.06	82.27	75.28	73.62	48.77	0.09
E/A ratio	2.71	2.23	2.73	2.61	3.41	0.50

[1] χ^2-test for categorical and ANOVA for continuous variables.

All hormone levels declined with age (Table 2). The correlation coefficients for all combined hormones was $r_s = -0.28$, while the individual correlations varied between $r_s = -0.14$ and -0.39. These relations were strongest for ADIOL and the two E_1 metabolites, followed by T, and weaker for E_2 and E_1. The 2/16 ratio was not significantly related to age. Only the A levels were associated with BMI; women with a higher BMI excreted more T and ADIOL. As a result, BMI showed a significant inverse relation with the E/A ratio. These associations did not change after excluding women on HRT, but they were stronger before menopause. Due to the small sample size, none of the associations between hormones and BMI were statistically significant after menopause. The correlation coefficients with T and ADIOL were slightly lower than in premenopausal women and, among those not using HRT, E_2 was weakly correlated with BMI ($r_s = 0.31$, $p = 0.08$). Percent MD was negatively related to age and BMI, but not for E or A levels. A weak positive association with 2-OH-E_1 disappeared after confounders adjustment, as did the correlation between the 2/16 ratio and MD was 0.07 ($p =$

0.22). Restricting the correlation analysis to premenopausal women did not change the results. After stratifying postmenopausal women by HRT use, MD was positively related to E_1, both OH metabolites, and T among women not taking HRT. The correlation coefficients were between 0.32 & 0.38, but were not statistically significant. However, postmenopausal women on HRT, E_1 and both OH metabolites were negatively associated with percent MD (r_s = -0.39).

Table 2. Relation of Hormones with Percent Densities, Age, and BMI.

Variable	Spearman Correlation Coefficients (*p*) with			
	Age	BMI	Percent Density	
			Unadjusted	Adjusted[1]
Age	--	-0.09	-0.21	--
		0.11	*0.0003*	
BMI	-0.09	--	-0.42	--
	0.11		*<.0001*	
E_1 (ng/ml)	-0.14	0.05	0.10	0.07
	0.02	*0.42*	*0.10*	*0.23*
E_2 (ng/ml)	-0.17	0.11	0.05	0.02
	0.004	*0.07*	*0.41*	*0.72*
2-OH-E_1 (ng/ml)	-0.32	-0.04	0.13	0.02
	<0.0001	*0.51*	*0.02*	*0.70*
16α-OH-E_1 (ng/ml)	-0.34	0.07	0.06	-0.04
	<0.0001	*0.23*	*0.30*	*0.48*
2/16 ratio	-0.09	-0.07	0.09	0.07
	0.12	*0.25*	*0.13*	*0.22*
T (ng/ml)	-0.20	0.27	-0.03	0.02
	0.0005	*<0.0001*	*0.57*	*0.77*
ADIOL (ng/ml)	-0.39	0.21	0.04	0.005
	<0.0001	*0.0002*	*0.49*	*0.92*
All hormones (ng/ml)	-0.28	0.09	0.08	0.03
	<0.0001	*0.11*	*0.14*	*0.62*
E/A ratio	0.04	-0.19	0.10	0.05
	0.48	*0.0007*	*0.08*	*0.44*

[1] Adjusted for age, menopausal status, HRT, BMI, ethnicity, age at menarche, age at first live birth, and number of children.

Mean levels of hormones by categories of percent MD (Figure 1) illustrate the lack of an association of MD with E_1, E_2, 2-OH-E_1, T, and ADIOL. The p-values for the respective trend tests were 0.94, 0.85, 0.54, 0.78, and 0.69. For 16α-OH-E_1, we observed a weak negative relation (p for trend = 0.19) that translated into a 2-5% higher 2/16 ratio for women in the highest MD category. The respective values for the five categories of percent MD were: 1.59, 1.68, 1.86, 1.85, and 1.99 with a p-value of 0.01 for the linear trend test.

This trend changed minimally when excluding the 37 women on HRT. However, stratification by menopausal status showed the association was stronger in pre- than postmenopausal women (p=0.08 vs. 0.97 for postmenopausal). HRT use did not affect the relation in postmenopausal women. Separate analysis for women with a BMI of ≤ 25 (p = 0.07) vs. women with a BMI ≥ 25 (p = 0.40) indicated that the relation was restricted to women with normal weight. Stratification by ethnicity showed similar trends in Caucasian and in Asian women (p = 0.07 and p = 0.04), but the mean 2/16 ratio was lower for Asians than for Caucasians in all MD categories.

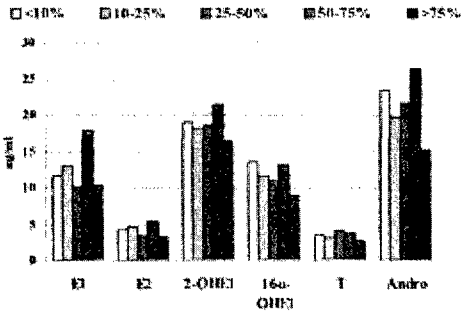

Figure 1. Levels of urinary hormones by categories of percent

Conclusions

In this cross-sectional investigation among women of different ethnicity, we observed higher percent MDs, lower urinary A levels, and a lower 2/16 ratio among Asian than Caucasian women. However, we did not observe any significant associations between urinary hormone levels and MD. The 2/16 ratio showed a relation with percent MD in a direction opposite to our initial hypothesis.

Women with percent MDs of 75% or greater had an approximately 25% higher 2/16 ratio than women with percent MDs below 10%. This relation was similar in Asian and Caucasian women, but it was not present among postmenopausal and overweight women. The ethnic differences in MD agree with previous studies (21, 31). Due to the smaller breast size of Asian women, percent MD is higher than in Caucasian women. However, the absolute MDs appears to be lower among Asian women (32).

Our findings agree with a previous report of MD and the 2/16 ratio (19). The study among postmenopausal women used a qualitative assessment method for MD assessment. The mean 2/16 ratio was 1.12 in the high risk group and 0.83 in the low risk group, a 35% difference. The results also agree with the higher 2/16 ratio in Finnish as compared to Asian women who have the lower BC risk (20). Although a number of studies have investigated the 2/16 ratios and BC, the evidence does not offer a definite answer to the question whether a higher 2/16 ratio reduces or increases BC risk. An association with postmenopausal BC was detected in one small case-control study (14), but a larger study did not support the hypothesis (33). Two cohort studies (17, 18) found a higher 2/16 ratio associated with a non-significantly reduced BC risk among premenopausal women. A recent study (16), conducted among Chinese women, reported a reduced BC risk with a higher urinary 2/16 ratio, but only when urine was collected prior to BC treatment.

The present study has a number of limitations. The strict eligibility criteria for the nutritional interventions may have introduced selection bias. The fact that our population was highly motivated, had a high proportion of subjects with a family history of BC (14.4%), and relatively few children suggests that the women in the study may have a higher than average risk to develop BC. Asian ethnicity at the age group of the women studied, at least second generation migrants, probably does not protect against BC. As shown in a large prospective study, BC incidence among Japanese women in Hawaii and California is at least as high as among Caucasians (34). The two urine collection protocols, overnight samples in premenopausal women vs. spot urine specimens in postmenopausal women, may have introduced additional bias. However, the possible effect on the results is difficult to assess. Although no information on urinary volume was collected, we are confident that the hormone concentrations reflect actual excretion patterns. For a subset of 196 premenopausal women who had creatinine levels available, we repeated the correlation analysis and adjusted for creatinine as a surrogate for urinary volume. None of the Es or As showed any association with percent MD.

Our results suggest an absence of a relationship between urinary sex steroids and MD. Given the negative results in studies of circulating endogenous hormones and MD (9, 10), it appears likely the effects of hormones are not mediated through MDs in premenopausal women. Unfortunately, the small number of postmenopausal women not taking HRT made it impossible to make any valid conclusions for that population. A larger study among women who have never taken HRT would be needed to elucidate this question in postmenopausal women. To understand the importance of different E metabolites in the etiology of BC may also require assessment of additional metabolites, such as 4-OH metabolites, as well as studies regarding the combined carcinogenic effects when different metabolites are present.

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Association of Genetic Polymorphisms with Serum Estrogens Measured Multiple Times During a 2-Year Period in Premenopausal Women

(Under review with Cancer Epidemiology, Biomarkers & Prevention)

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Abstract

There is evidence that circulating estrogens are associated with breast cancer risk. In this study of premenopausal women, we explored the association of polymorphisms in genes in the estrogen synthesis and metabolism pathways with serum and urinary levels of estrone (E1) and estradiol (E2) and with the urinary ratio of 2-hydroxyestrone (2-OHE1)/16α-hydroxyestrone (16α-OHE1). This analysis included 220 women, who were participants in a 2-year randomized soy dietary intervention. Blood specimens were collected in the luteal phase of the menstrual cycle an average of 4.4 times over 2 years. Overnight urinary specimens were collected on the same cycle day, only at baseline. Levels of E1, E2, 2-OHE1, and 16α-OHE1 were measured by enzyme immunoassays. The DNA samples were analyzed by PCR/RFLP for the *COMT* Val158Met, *CYP1A1*2A*, *CYP1A1*2B*, *CYP1A2*1F*, *CYP1B1* Val432Leu, and *CYP17* T27C polymorphisms. We applied mixed models to investigate the relations between genotypes and repeated serum hormone measurements and generalized linear models to assess associations between genotypes and urinary estrogen metabolites. The *CYP1A2* C allele was significantly associated with lower serum E2 levels; in CC genotype carriers, serum E2 levels were 26.3% lower than in homo- and heterozygous common allele carriers combined ($p = 0.01$). *CYP1A2*1F* also affected the urinary 2OHE1/16α-OHE1 ratio; carriers of the variant C allele had a markedly lower ratio than individuals with the AA genotype (1.37 *versus* 1.76; $p = 0.002$). These data suggest that *CYP1A2*1F* is associated with lower circulating levels of E2, and that it may be a susceptibility locus for breast cancer.

Introduction

There is persuasive evidence from epidemiological studies and experimental models for a role of endogenous estrogens and their metabolites in the pathogenesis of breast cancer. The most widely accepted theory states that estrogens increase the rate of mammary epithelial cell proliferation by stimulating estrogen receptor-mediated transcription (1-4). Another hypothesis is that estradiol increases risk through genotoxic metabolites that directly damage DNA (1, 2, 5-8). Epidemiological studies showed that women with elevated circulating E2 levels are at higher risk for breast cancer (9-15). The major pathways of endogenous metabolism of estrogens involve hydroxylation at either C2 (2-OHE1) or C16 (16α-OHE1). The 2-OHE1 metabolite has little estrogenic activity, whereas 16α-OHE1 is an estrogen agonist (16-19). In a study by Ho *et al.* (20), 16α-OHE1 was shown to initiate neoplastic transformation of mammary epithelial cells. Other experimental studies showed an anti-estrogenic role of 2-OHE1 (21) and a genotoxic effect of 16α-OHE1 (22-24). An inverse association between the 2-OHE1/16α-OHE1 ratio and breast cancer risk has also been reported in several epidemiological studies (18, 19, 25).

Genes involved in estrogen synthesis and metabolism have been intensively studied in recent years in relation to breast cancer risk. However, few studies investigated associations between genetic polymorphisms and plasma estrogen levels, and only two studies explored polymorphisms in estrogen metabolism pathway genes and urinary 2-OHE1/16α-OHE1 ratio. Feigelson *et al.* (26) first reported an association between the *CYP17* T27C variant and increased plasma estradiol levels in 83 nulliparous, ovulating, young women sampled either during the luteal phase or the follicular phase of the menstrual cycle; although, the association for the follicular samples was of borderline significance only. Significant associations between the *CYP17* C allele and increased serum estrogen levels were also found among postmenopausal women (27, 28). However, other studies failed to show a relation (29-32). The association between serum sex hormones and *CYP1B1* Val432Leu and *COMT* Val158Met was investigated among premenopausal women by Garcia-Closas *et al.* (29) and among postmenopausal women by Tworoger *et al.* (32) and Dunning *et al.* (33). Premenopausal hetero- and homozygous carriers of the Val432Leu variant allele had significantly increased luteal E2 levels; no association was found among postmenopausal women. The role of the *COMT* Val158Met polymorphism on serum E2 levels was also studied by Worda *et al.* (34) in an intervention study (oral administration of 2 mg of E2 valeriate); 3 hours after administration, serum E2 levels were significantly higher in women with the Met/Met genotype.

Tworoger *et al.* (32) investigated the relationship between several polymorphisms and urinary estrogen metabolites and found significantly higher 16α-OHE1 levels in postmenopausal women with the COMT Met/Met genotype, compared to those with the Val/Val genotype. The *CYP1A1*2A* polymorphism was found to alter 2-OHE1/16α-OHE1 ratio in the study by Taioli *et al.* CYP1A2 activity was found to be negatively associated with free estradiol concentrations in the recent study by Hong *et al.* (35). In a recent study by our group (36), we reported lower mammographic density for women carrying the *CYP1A2* variant C allele; however, this was the opposite of our hypothesis as carriers of this allele have been shown to have lower CYP1A2 inducibility (37). To our knowledge, *CYP1A2*1F* polymorphism has not previously been studied in relation to either breast cancer risk or estrogen concentrations.

The conflicting results of past studies on genetic polymorphisms and estrogen levels may be explained in part by the strong temporal fluctuation of serum E1 and E2 levels (11, 38). In addition, the origin of estrogens differs between pre- and postmenopausal women and, therefore, different associations may be expected by menopausal status. In the present study, we investigated the association of several polymorphisms in estrogen synthesis and metabolism pathway genes (*COMT* Val158Met, *CYP1A1*2A*, *CYP1A1*2B*, *CYP1A2*1F*, *CYP1B1* Val432Leu, and *CYP17* T27C) with serum E1 and E2 levels in healthy premenopausal women with regular menstrual cycles who participated in dietary intervention trial and had their estrogen levels measured several times over 2 years. It is plausible that these polymorphisms can affect serum estrogen levels and distribution of estrogen metabolites in urine by changing activity of important enzymes in estogen synthesis and metabolism pathways. Because estrogens were measured multiple times over 2 years, we had a more reliable measure than the single sample uniformly used in past studies. We also investigated the association between these polymorphisms and urinary 2-OHE1/16α-OHE1 ratio which is thought to be a stable marker with relatively moderate within-person variation (39).

Methods

Study Design and Population. This cross-sectional study included 220 healthy premenopausal women recruited at mammography clinics who participated in a 2-year randomized controlled soy dietary intervention (40). The inclusion criteria were having a normal mammogram at baseline and no previous history of cancer, having regular menstrual cycles and an intact uterus and ovaries, not taking oral contraceptives or other hormones, not being pregnant or lactating, and consuming less than 7 servings of soy a week. All subjects completed a validated food frequency questionnaire

(41), as well as a detailed questionnaire on reproductive, medical, and anthropometric factors at baseline. Height and weight measurements were taken at each blood collection. Subjects in the intervention group consumed two daily servings of soy food containing 50 mg of isoflavons, whereas the control subjects maintained their regular diet. Subject recruitment, samples collection, and intervention results are described in detail in ref. (40). Because the trial showed no significant intervention effect on any of the serum hormones during 2-year period, we combined the data from both study groups for the current study. The protocol was approved by the Committee on Human Studies of the University of Hawaii and by the Institutional Review Boards of the three hospitals where women were recruited, and written informed consent was obtained from all participants.

Samples Collection. Fasting blood and overnight urine samples were collected between 7:30 and 10:00 a.m. for 208 and 184 women, respectively, during the luteal phase of a menstrual cycle, on the 5th day after ovulation, corresponding to approximately day 19 of the menstrual cycle. For less than 1% of women, blood samples were obtained on day 4 or 6. The sampling day was determined by using an ovulation test that detects the time of ovulation by measuring luteinizing hormone (LH) in a urine sample (42) and confirmed by serum progesterone values > 5 ng/ml. In addition to baseline, blood samples were obtained in the same manner at approximately 3, 6, 12, and 24 months. During 2-year study period, information on the menstrual cycles was carefully recorded and updated at each contact with study participants.

Hormone measurements. Serum estrone (E1) and estradiol (E2) levels were measured in all samples by radioimmunoassays (RIA) in the laboratory of Dr. Stanczyk (University of Southern California, Los Angeles, Ca, USA) as described previously (43). Inter-assay coefficients of variation (CVs) for the serum estrogens were 17.7% for E1 and 11.2% for E2. Urinary E1, E2, 2-OHE1, and 16α-OHE1 were measured in the laboratory of Dr. Kaaks (International Agency of Research on Cancer, Lyon, France), using a previously published protocol (42). All measurements were done in duplicate, including hydrolysis, solid phase extraction, HPLC, and RIA steps. For quality control, 2 control samples containing known amounts of steroids were included for all the analysis steps in each analytical batch. The detection limits for urine E1 and E2 were 2.0 ng/100ml. Intra- and inter-batch CVs were 4.7% and 16%, respectively, for E1 (at a concentration of 3300 ng/l), and 1.7% and 14% for E2 (at 320 ng/l). The metabolites, 2-OHE-1 and 16α-OHE-1, were measured by solid-phase enzyme immunoassays after enzymatic hydrolysis with Helix Pomatia (Estramet, Immunacare Corporation, Bethlehem, USA). Mean intra-batch and inter-batch CVs were 10% and 15%,

respectively, for both analytes. E1, E2, 2-OHE1, and 16α-OHE1 in urine were adjusted for urinary creatinine levels.

Genetic Analysis. DNA was purified from whole blood using Qiagen Midi Kits (Qiagen, Valencia, CA). The DNA samples were analyzed by PCR/RFLP for the *COMT* Val158Met, *CYP1A1*2A, CYP1A1*2B*, CYP1A2**1F* (rs762551), *CYP1B1* Val432Leu, and *CYP17* T27C (rs743572) polymorphisms, as reported previously (44, 45). In addition, we genotyped the samples for the rare *CYP1A1*4* variant in order to correctly assign *CYP1A1*2B* genotypes (46). Forty-two percent of the samples were assayed twice for quality control. The concordance rates among duplicates were 100% for *CYP1A1*2*, *CYP1A2*F*, and *COMT*, 99.9% for CYP17, and 91.4% for *CYP1A1*3* and *CYP1B1*.

Statistical Analysis. The SAS statistical software package version 8.2 (SAS Institute Inc., Cary, NC) was used for the data analysis. In the questionnaire, subjects marked all ethnic backgrounds that applied to their parents. We assigned summary categories according to the following rules. A woman was classified as Caucasian if both of her parents were of Caucasian ancestry. In agreement with a common rule applied in the State of Hawaii (47), women with any Hawaiian heritage were classified as Native Hawaiian. If subjects reported two or three ethnic backgrounds, they were classified according to the ethnicity shared by both of the parents, or the ethnicity of the mother when parents did not share any ethnic background. Because of the similarity in variant allele frequencies, Japanese, Chinese, and Filipino women were combined into the Asian category. Because of their mixed ancestries, the Native Hawaiian women (N = 26) were combined into the Mixed category that also included women with more than 3 ethnic backgrounds. Body mass index (BMI) was calculated as the ratio of weight in kilograms divided by the square of the height in meters. All outcome and dietary variables, as well as BMI, were transformed using their natural logarithm because they were not normally distributed.

We used mixed general linear models (PROC MIXED SAS procedure) with repeated measures of E1 and E2 (random effect) to model the fixed effects of genotype, time of blood draw, study group assignment (intervention versus control), interaction between genotype and study group, and interaction between study group and time (to explore differences in the intervention effect on hormone levels at different times during the 2-year study period). Given the lack of an intervention effect and no indication of effect modification by genotype or time on circulating hormones, study

group, time, and both interaction variables were dropped from the final models. The PROC GLM procedure was used to assess associations of urinary E1, E2, 2-OHE1, and 16α-OHE1 with genotype. The PROC MIXED SAS procedure was also used to calculate intra-class correlations between the repeated measures of serum E1 and E2 levels.

In preliminary analyses, we assessed the relationships between each of the outcome variables with age, ethnicity, BMI, age at menarche, age at first live birth, parity, family history of breast cancer, total daily calories, total daily calories from fat, daily alcohol, fat, saturated fat, and caffeine intakes and explored associations between each polymorphism and ethnicity, age at menarche, and BMI. We also introduced these variables into the mixed models to look for potential confounders. Our final models included covariates that were associated with at least one of the predictor or outcome variables, namely: ethnicity, age, BMI, age at menarche, and number of children. As no significant changes occurred in BMI over time, a baseline BMI measurement was included into the models. We also explored associations between genotypes and hormonal variables within each ethnic group.

For all models, genotype was successively treated as a non-ordered categorical variable to obtain least-square means and to test for heterogeneity, and as an ordered categorical variable (0, 1 and 2, for the homozygous common genotype and the hetero- and homozygous variant genotype, respectively) to test for a gene dosage effect. In addition, we evaluated genotype as a dichotomous variable, combining hetero- and homozygous variant allele carriers for a pair-wise comparison with homozygous carriers of the common allele (testing a dominant pattern of inheritance) and combining homo- and heterozygous common allele carriers for a pair-wise comparison with homozygous variant allele carriers (testing a recessive genetic model).

Results

Characteristics of the Study Population. The subject characteristics are presented in Table 1 by ethnicity. The mean age of the study participants was 43.0 years (SD, 2.8; range 34-47). The sample included almost equal number of Caucasian and Asian women. On average, women of mixed ethnicity were slightly younger, and Caucasians had a somewhat older age at menarche, lower parity, and higher age at first live birth. BMI was lowest among Asian women, intermediate in Caucasians, and highest in women of mixed ethnicity ($p = 0.048$). Few women (6%) reported to be current smokers.

The distributions of the genotypes were all consistent with Hardy-Weinberg equilibrium within each ethnic group. The variant allele frequencies were significantly different by ethnicity for all polymorphisms under study, except for *CYP17* T27C and *CYP1A2*1F*.

Out of the 208 women who donated blood samples during the 2-year study period, 116 women had serum E1 and E2 levels measured 5 times, whereas 56 women had 4 measurements, 24 had 3 measurements, and 9 women had 2 measurements. Overall, serum estrogens were measured 4.4 times. The variability in number of measurements was due to women dropping out of the study (31 subjects) and to sample exclusions due to wrong timing (16 samples), or occasional use of exogenous hormones (72 samples). Overall, we had 894 measurements for each E1 and E2, representing 86% of the maximum possible number of measurements (n = 1,040).

The within-person intra-class correlation for E1 and E2 over time was 0.55 and 0.41, respectively. In Table1, these hormonal measurements were averaged for each subject over the number of collections (Table 1). Overall, mean serum E1 and E2 levels were lowest in Asian women and highest in women of mixed ethnicity; these differences, however, were not statistically significant. The mean urinary levels of E1 and E2 were also similar among ethnic groups (Table 1). However, statistically significant differences were observed in the mean urinary 2-OHE1/16α-OHE1 ratio, with the highest value for Caucasians and the lowest for Asians, reflecting a significantly higher 2-OHE1 excretion in Caucasian women. Adjustment for BMI, age, time of blood draw, intervention, and other variables under study did not change these findings.

Relationships Among Covariates: We did not find any significant associations between serum E1 and E2 and all covariates under study, except for an inverse association of serum E1 levels with parity ($p = 0.03$) and of E2 levels with BMI ($p = 0.049$). When stratified by ethnicity, E2 levels in Asian subjects were inversely associated with age ($p = 0.02$). Urinary 2-OHE1 also showed a significant inverse association with parity ($p = 0.03$) and BMI ($p = 0.05$), and a direct association with ethanol consumption ($p = 0.04$). CYP17 variant allele carriers had significantly younger age at menarche (p for trend = 0.02). This association remained significant after adjusting for BMI, but was no longer significant after adjusting for ethnicity ($p = 0.056$). When examined by ethnic group, this association between *CYP17* and age at menarche was statistically significant in Caucasian women only ($p = 0.02$).

Polymorphisms and Serum E1 and E2. We did not find any significant associations between the genotypes under study and serum E1 levels before (Table 2) and after (Table 3) adjusting for covariates. *CYP1A2*1F* was the only polymorphism associated with serum E2 levels (Table 2). This inverse association remained significant after adjusting for ethnicity and other covariates (Table 3). Further inclusion into the mixed model of the study group assignment, time of the blood collection, total daily calories, total daily calories form fat, daily consumption of fat, saturated fat, alcohol, and caffeine did not affect these relationships. Subjects with the *CYP1A2* CC genotype had mean serum E2 levels that were 25.8% lower than subjects with the AA genotype and 29.7% lower than subjects with the AC genotype (p for pair-wise comparison = 0.01 and 0.003, respectively). Moreover, homozygous variant alelle carriers had 26.3% lower mean serum E2 levels than homo- and heterozygous common allele carriers combined (p for recessive model = 0.01). When stratified by ethnicity, lower mean E2 levels were observed in subjects with the CC genotype in all 3 ethnic groups, reaching statistical significance in Caucasians (Table 4).

Polymorphisms and Urinary E1 and E2. We did not observe any significant associations between single urinary E1 and E2 measurements and any of the polymorphisms under study (data not shown).

Polymorphisms and Urinary 2-OHE1/16α-OHE1. Tables 5 and 6 show the associations between the polymorphisms under study and urinary levels of 2-OHE1 and 16α-OHE1, as well as their ratio, before and after adjustment for covariates. A significantly lower mean ratio was observed in women with the *CYP1A2* AC genotype compared to women with the AA or CC genotype (p for heterogeneity = 0.003), resulting from a higher 16α-OHE1 excretion (p for heterogeneity = 0.007). Moreover, prior to adjustment for covariates, the mean 2-OHE1/16α-OHE1 ratio was greater (1.87 *versus* 1.53) for homozygous *COMT* Met allele carriers compared to women with the Val/Val and Val/Met genotypes combined (p=0.07), due to significantly higher levels of 2-OHE1 (p for trend = 0.008; p for a recessive model = 0.004). However, this association was no longer significant after adjustment other covariates (p = 0.11).

Discussion

In this study, we found an association between *CYP1A2*1F* and both serum E2 levels and the urinary 2-OHE1/16α-OHE1 ratio, providing evidence for a role of this polymorphism in the

regulation of circulating E2 levels and its metabolism. No significant association was observed between *CYP1A2*1F* and serum E1 or urinary E1 and E2 levels. Our study also found no convincing evidence for an effect of the other polymorphisms on serum E1 and E2 levels, their excretion in urine, or the urinary 2-OHE1/16α-OHE1 ratio. An effect of the *COMT* Val158Met polymorphism on urinary 2-OHE1 excretion was observed, but this association was no longer present after adjustment for ethnicity.

CYP1A2 is thought to be the most important enzyme in the 2-hydroxylation of E1 and E2 (48). This enzyme is known to be inducible, and previous studies have shown variation in activity level by sex, age, race, smoking status, coffee and alcohol consumption, and exposure to various combustion products and contaminants (e.g. dioxin) [reviewed in ref. (49)]. In our study, we did not have an opportunity to investigate the effect of smoking on the relationship between *CYP1A2*1F* and serum E2 as few women were smokers; however, alcohol and coffee consumption did not influence this relationship. Our finding of lower estradiol levels in carriers of *CYP1A2*1F* polymorphism is consistent with the lower mammographic density in subjects with this genotype, reported in recent study by our group (36). We are not aware of any other studies of this polymorphism in relation to circulating estrogen levels or the urinary 2-OHE1/16α-OHE1 ratio.

COMT catalyzes the conjugation of catecholestrogens, resulting in their conversion into monomethylethers. Studies have shown that individuals with the Met/Met genotype have 2 to 3-fold decreased activity of this enzyme (50), and that this phenotype is inherited as an autosomal recessive trait (51). Increased mean levels of 2-OHE1 in homozygous *COMT* Met allele carriers in our study are in agreement with both of these findings and, also, confirm the results obtained by Tworoger *et al.* (32) in postmenopausal women. However, in our study, the association was not significant after adjustment for ethnicity.

Higher serum levels of E2 measured during the luteal phase of the menstrual cycle were found in *CYP17* A27C allele carriers by Feigelson *et al.* (26). Other studies of premenopausal women failed to produce evidence to support this finding (29). Consistent with these data, we found no association between the *CYP17* 27C allele and either serum or urine estrogens or the urinary 2-OHE1/16α-OHE1 ratio. The only previous study that investigated estrogens and polymorphisms in premenopausal women other than *CYP17* observed an association between the *CYP1B1* 432Leu allele and increased serum luteal E2 levels (29). In contrast, we found no association with this

genetic variant. This inconsistency might be explained by differences in study populations: our subjects were significantly older and more ethnically diverse.

Most importantly, our study used repeated measures of E1 and E2 levels over a 2-year period, in contrast to the one-time assessment universally used in past studies. Multiple measurements are likely to greatly increase the reliability of the hormone measurements, and, thus, the statistical power, by decreasing the intra-individual variation in serum estrogen levels, which is known to be substantial (38). The fact that ovulation was confirmed by progesterone measurements and that sampling was narrowly timed in the luteal phase of the menstrual cycle also adds confidence to the standardization of the hormonal assessment. Although the urinary 2-OHE1/16α-OHE1 ratio was only measured once in our study, the within-person variability of this ratio over a 2-month period in Caucasian women has been shown to be moderate, so that a single urine sample is an adequate predictor of long-term level (39). The data by Westerlind *et al.* (52) also suggested that a first-morning void (and, thus, an overnight sample as in our study) is representative of a 24-hour collection and that the 2-OHE1/16α-OHE1 ratio is relatively constant throughout a 24-hour period.

Another strength of the present study is that we only included women who had regular menstrual cycles and who were not taking hormonal contraceptives. We also carefully collected data on reproductive history and nutrition to adjust estrogen levels for potential confounders. Moreover, the precision of the hormonal measurements was monitored using internal standards and the laboratory reproducibility of these measurements was satisfactory. Finally, the genotype distributions in our study were in agreement with previous reports (53), suggesting that our sample is representative of the general population. The main limitation of our study was that the sample size was not large enough to test for gene-gene and gene-environment interactions.

A life-long exposure to modestly increased estrogen levels could produce a substantial cumulative effect on disease risk. For example, a model proposed by Pike *et al.* (54) demonstrated that a 20% difference in circulating estrogen may result in a more than 2-fold increase in lifetime breast cancer risk. We found that carriers of the *CYP1A2* CC genotype had a consistently 26.3% lower estradiol levels. If these levels measured during a 2-year period were representative of lifetime patterns, these women may be at 50% lower risk of breast cancer. The present study suggests that the *CYP1A2*1F* allele should be studied in relation to breast cancer susceptibility.

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Table 1. Characteristics of the study population by ethnicity

Variables	Asian N = 85	Caucasian N = 83	Mixed N = 52	*P**
Age (years)	43.5 (42.9-44.1)	42.9 (42.3-43.5)	42.4 (41.6-43.2)	*0.11*
BMI (kg/m^2)	24.6 (23.5-25.6)	25.8 (24.7-26.9)	26.7 (25.3-28.2)	***0.048***
Family history of breast cancer (%)	11.0	17.1	15.7	*0.52*
Age at menarche before 13 (%):	60.0	51.8	67.3	*0.19*
Number of children (%):			17.3	
none	23.5	34.9	17.3	
1-2	57.7	44.6	51.9	*0.10*
3 or more	18.8	20.5	30.9	
Estrogen levels in serum (means of multiple measurements over 2 years):				
E1 (pg/ml)	84.7 (77.5-92.7)	86.6 (79.0-95.0)	91.5 (81.7-102.6)	*0.56*
E2 (pg/ml)	129.0 (118.1-140.9)	135.1 (123.5-147.9)	141.4 (126.3-158.2)	*0.58*
Urinary estrogens and their metabolites (measured once at baseline):				
E1 (ng/mg creatinine)	8.7 (7.2-10.4)	9.4 (7.8-1.2)	9.1 (7.2-11.4)	*0.83*
E2 (ng/mg creatinine)	3.7 (3.2-4.3)	3.6 (3.1-4.2)	3.7 (3.1-4.4)	*0.97*
2-OHE1 (ng/mg creatinine)	20.2 (17.2-23.7)	27.1 (23.2-31.7)	18.6 (15.3-22.6)	***0.006***
16α-OHE1 (ng/mg creatinine)	15.0 (13.0-7.4)	14.4 (12.5-16.5)	12.9 (10.8-15.4)	*0.42*
2-OHE1/16α-OHE1 ratio	1.35 (1.19-1.54)	1.89 (1.66-2.15)	1.51 (1.28-1.78)	***0.008***
Variant allele frequencies:				
COMT 158Met	0.25	0.57	0.22	***<0.0001***
*CYP1A1**2A	0.44	0.13	0.41	***<0.0001***
*CYP1A1*2B*	0.30	0.07	0.23	***<0.0001***
*CYP1A2*1F*	0.27	0.22	0.27	*0.07*
CYP1B1 432Leu	0.19	0.46	0.33	***<0.0001***
CYP17 27C	0.48	0.35	0.39	*0.10*

NOTE: The values are means for age and geometric means (95% confidence intervals) for other variables, except when indicated.
**p* values from ANOVA for continuous variables and from chi-square test for categorical variables.

Table 2. Unadjusted geometric mean (95% confidence intervals) serum E1 and E2 measured over a 2-year period by genotype.

Genotype	N	E1 (pg/ml)		E2 (pg/ml)	
		Mean (95% CI)	*p*	Mean (95% CI)	*p*
COMT					
Val/Val	84	80.4 (74-87.3)		123.7 (114.1-134.1)	
Val/Met	83	86.8 (79.9-94.3)	*0.42**	131.3 (121.1-142.2)	*0.59**
Met/Met	31	81.6 (71.3-93.5)	*0.56*†	126.3 (110.7-144.1)	*0.57*†
CYP1A1*2A					
m1/m1	100	81.1 (75.2-87.5)		124.7 (115.8-134.2)	
m1/m2	71	84.5 (77.2-92.4)	*0.53**	128.5 (117.8-140.3)	*0.74**
m2/m2	26	88.8 (76.5-103.1)	*0.26*†	132.2 (114.3-153.0)	*0.43*†
CYP1A1*2B					
AA	130	80.6 (75.4-86.1)		124.2 (116.5-132.5)	
AG	58	88.4 (80.1-97.6)	*0.26**	133.4 (121.1-146.9)	*0.46**
GG	10	89.3 (70.4-113.4)	*0.12*†	131.6 (104.1-166.3)	*0.26*†
CYP1A2*1F					
AA	110	82.1 (76.4-88.1)		126.4 (118.0-135.3)	
AC	76	86.9 (79.7-94.8)	*0.25**	133.9 (123.3-145.4)	***0.02****
CC	12	72.1(58.0-89.7)	*0.97*†	97.3 (79.0-119.8)	*0.45*†
AA+AC	186	84.0 (67.5-104.5)	*0.18*‡	129.4 (122.7-136.4)	***0.01***‡
CYP1B1					
Val/Val	91	80.3 (74.2-86.9)		131.2 (121.5-141.7)	
Val/Leu	86	85.2 (78.5-92.4)	*0.45**	125.4 (115.9-135.7)	*0.44**
Leu/Leu	21	88.3 (75.0-104.10	*0.21*†	117.9 (100.8-137.9)	*0.21*†
CYP17					
TT	70	87.2 (79.7-95.5)		128.3 (117.5-140.1)	
TC	96	81.9 (75.8-88.4)	*0.41**	131.1 (121.7-141.2)	*0.18**
CC	32	79.1 (69.3-90.3)	*0.19*†	114.1 (100.3-129.8)	*0.25*†

**p* global test for comparison of means; $^{\dagger}p$ for gene dosage effect; $^{\ddagger}p$ for testing a recessive genetic model.

Table 3. Geometric mean (95% confidence intervals) serum E1 and E2 measured over a 2-year period, adjusted in a mixed models for ethnicity, age, BMI, age at menarche, and parity by genotype

Genotype	N	E1 (pg/ml)		E2 (pg/ml)	
		Mean (95% CI)	*p*	Mean (95% CI)	*p*
COMT					
Val/Val	84	80.1 (73.6-87.3)		123.9 (114.0-134.7)	
Val/Met	83	88.0 (80.9-95.9)	*0.32**	133.2 (122.6-144.7)	*0.49**
Met/Met	31	84.3 (72.7-97.8)	*0.34*†	128.4 (111.1-148.4)	*0.47*†
CYP1A1*2A					
m1/m1	100	81.7 (75.3-88.5)		124.7 (115.3-134.9)	
m1/m2	71	85.4 (77.6-93.8)	*0.62**	131.4 (119.9-144.1)	*0.66**
m2/m2	26	88.6 (76.1-103.2)	*0.33*†	132.0 (113.8-153.1)	*0.41*†
CYP1A1*2B					
AA	130	81.0 (75.6-86.8)		125.1 (116.9-133.8)	
AG	58	89.6 (81.0-99.2)	*0.27**	134.9 (122.2-149.0)	*0.47**
GG	10	88.0 (69.0-112.3)	*0.15*†	130.6 (102.8-165.9)	*0.30*†
CYP1A2*1F					
AA	110	82.6 (76.8-88.9)		128.3 (119.6-137.5)	
AC	76	88.6 (81.1-96.9)	*0.14**	135.4 (124.3-147.5)	***0.01****
CC	12	70.7 (56.9-87.8)	*0.95*†	95.2 (77.3-117.2)	*0.31*†
AA + AC	186	84.0 (79.5-88.7)	*0.16*‡	129.2 (122.6-136.2)	***0.01***‡
CYP1B1					
Val/Val	91	81.2 (74.4-88.2)		133.5 (123.1-144.8)	
Val/Leu	86	85.5 (78.7-92.8)	*0.53**	124.4 (114.9-134.7)	*0.42**
Leu/Leu	21	89.4 (75.3-106.3)	*0.26*†	122.4 (103.5-144.8)	*0.22*†
CYP17					
TT	70	87.2 (79.7-95.5)		128.3 (117.5-140.2)	
TC	96	84.0 (77.6-91.0)	*0.31**	132.2 (122.3-142.8)	*0.33**
CC	32	76.5 (66.7-87.9)	*0.14*†	117.4 (102.6-134.3)	*0.46*†

**p* global test for comparison of means; $^{\dagger}p$ for gene dosage effect; $^{\ddagger}p$ for testing a recessive genetic model.

Table 4. Geometric mean (95% confidence intervals) serum E2 before and after adjustment in mixed models for age, BMI, age at menarche, and parity by ethnicity and *CYP1A2*1F* genotype

*CYP1A2*1F*	N	E2 (pg/ml) unadjusted		E2 (pg/ml) adjusted	
		Mean (95% CI)	*p*	Mean (95% CI)	*p*
Asian					
AA	38	132.8 (119.3-147.8)		132.6 (119.4-147.4)	
AC	37	123.1 (110.2-137.6)	*0.01**	124.5 (11.6-139.0)	*0.13**
CC	3	87.1 (59.4-127.6)	*0.10*†	88.5 (60.3-130.0)	*0.11*†
AA+AC	75	127.9 (118.4-138.1)	*0.06*‡	128.6 (119.3-138.6)	*0.06*‡
Caucasian					
AA	47	116.5 (106.0-128.1)		117.1b(106.4-128.9)	
AC	28	144.4 (127.2-163.9)	***0.008****	143.6 (126.3-163.4)	***0.009****
CC	3	87.7 (60.3-127.5)	*0.025*†	85.3 (58.2-124.9)	*0.35*†
AA+AC	75	137.7 (121.6-155.9)	***0.04***‡	136.7 (120.4-155.2)	*0.06*‡
Mixed					
AA	29	133.9 (115.1-155.8)		136.8 (117.2-159.7)	
AC	14	141.9 (115.5-174.3)	*0.79**	140.9 (114.4-173.5)	*0.64**
CC	6	125.1 (91.6-170.9)	*0.95*†	117.7 (85.8-161.4)	*0.62*†
AA+CC	43	137.5 (115.9-163.1)	*0.80*‡	134.5 (113.1-160.1)	*0.92*‡

**p* global test for comparison of means; $^{\dagger}p$ for gene dosage effect; $^{\ddagger}p$ for testing a recessive genetic model.

Table 5. Unadjusted geometric mean (95% confidence intervals) urinary estrogen metabolites (measured at base line by genotype

Genotype	N	2-OHE/16-OHE Ratio		2-OHE1 (ng/mg creatinine)		16α-OHE1 (ng/mg creatinine)	
		Mean (95% CI)	*p*	Mean (95% CI)	*p*	Mean (95% CI)	*p*
COMT							
Val/Val	75	1.50 (1.32-1.71)		20.0 (13.8-29.0)		13.7 (11.9-15.7)	
Val/Met	74	1.55 (1.36-1.76)	*0.18**	21.5 (14.8-31.2)	***0.01****	14.0 (12.2-16.0)	*0.36**
Met/Met	31	1.87 (1.53-2.29)	*0.10*†	30.6 (21.1-44.4)	***0.008***†	16.4 (13.2-20.2)	*0.22*†
Val/Val/+Val/Met	149	1.53 (1.39-1.67)	*0.07*‡	20.7 (16.3-26.3)	***0.004***‡	13.8 (12.6-15.2)	*0.16*‡
CYP1A1*2A							
m1/m1	88	1.60 (1.42-1.80)		23.4 (20.3-27.0)		14.6 (13.4-16.0)	
m1/m2	67	1.63 (1.42-1.87)	*0.53**	21.8 (18.5-25.7)	*0.35**	13.5 (12.4-14.8)	*0.71**
m2/m2	24	1.40 (1.12-1.76)	*0.47*†	18.7 (14.3-24.6)	*0.16*†	14.5 (13.3-15.9)	*0.71*†
CYP1A1*2B							
AA	117	1.61 (1.45-1.78)		22.3 (19.7-25.2)		13.8 (12.4-15.4)	
AG	54	1.56 (1.34-1.81)	*0.69**	22.2 (18.5-26.6)	*0.91**	14.4 (12.3-16.9)	*0.35**
GG	9	1.36 (0.94-1.98)	*0.44*†	20.1 (13.1-30.9)	*0.75*†	18.7 (12.6-27.7)	*0.24*†
CYP1A2 *1F							
AA	100	1.79 (1.60-1.99)		22.6 (19.8-25.8)		13.0 (11.6-14.6)	
AC	70	1.33 (1.17-1.51)	***0.003****	22.4 (19.1-26.2)	*0.39**	16.8 (14.7-19.3)	***0.004****
CC	10	1.61 (1.14-2.27)	***0.008***†	16.5 (10.8-25.4)	*0.37*†	10.3 (7.1-14.8)	*0.25*†
AC + CC	80	1.36 (1.21-1.53)	***0.001***§	21.6 (18.6-25.0)	*0.65*§	15.9 (13.9-18.0)	***0.03***§
CYP1B1							
Val/Val	79	1.49 (1.32-1.69)		27.7 (19.5-26.4)		15.4 (13.5-17.5)	
Val/Leu	83	1.62 (1.44-1.84)	*0.41**	21.3 (18.4-24.7)	*0.78**	13.5 (11.8-15.3)	*0.31**
Leu/Leu	18	1.78 (1.38-2.30)	*0.18*†	23.4 (17.1-31.9)	*0.37*†	13.1 (10.0-17.2)	*0.15*†
CYP17							
TT	66	1.55 (1.35-1.77)		21.1 (17.9-24.9)		14.1 (12.2-16.3)	
TC	89	1.67 (1.48-1.87)	*0.89**	23.3 (20.2-26.9)	*0.59**	14.1 (12.5-16.0)	*0.33**
CC	25	1.39 (1.12-1.73)	*0.70*†	20.8 (16.0-27.2)	*0.79*†	15.0 (11.9-18.9)	*0.71*†

**p* global test for comparison of means; $^{\dagger}p$ for gene dosage effect; $^{\ddagger}p$ for testing a recessive genetic model; $^{\S}p$ for testing a dominant genetic model.

Table 6. Geometric mean (95% confidence intervals) urinary estrogen metabolites (measured at base line, adjusted in a mixed models for age, ethnicity, BMI, age at menarche, and parity by genotype

Genotype	N	2-OHE/16-OHE Ratio		2-OHE1 (ng/mg creatinine)		16α-OHE1 (ng/mg creatinine)	
		Mean (95% CI)	*p*	Mean (95% CI)	*p*	Mean (95% CI)	*p*
COMT							
Val/Val	*75*	1.58 (1.39-1.80)		20.8 (17.8-24.2)		13.5 (11.7-15.5)	
Val/Met	74	1.55 (1.36-1.76)	*0.89**	21.2 (18.2-24.8)	*0.27**	13.9 (12.0-16.0)	*0.38**
Met/Met	31	1.63 (1.32-2.02)	*0.91*†	26.5 (20.5-34.3)	*0.19*†	16.4 (13.0-20.7)	*0.23*†
Val/Val+Val/Met	149	1.56 (1.43-1.71)	*0.69*‡	21.0 (18.9-23.4)	*0.11*‡	13.7 (12.4-15.1)	*0.17*‡
CYP1A1* 2A							
m1/m1	88	1.50 (1.33-1.69)		19.7 (16.4-23.6)		14.6 (12.7-16.7)	
m1/m2	67	1.72 (1.50-1.97)	*0.32**	21.1 (17.8-25.0)	*0.75**	13.3 (11.4-15.4)	*0.65**
m2/m2	24	1.54 (1.23-1.93)	*0.47*†	19.0 (14.4-25.3)	*0.95*†	14.4 (11.2-18.6)	*0.71*†
CYP1A1 *2B							
AA	117	1.55 (1.40-1.72)		21.1 (18.6-23.9)		13.6 (12.2-15.3)	
AG	54	1.65 (1.42-1.92)	*0.73**	23.3 (19.5-27.9)	*0.68**	14.3 (12.1-16.9)	*0.33**
GG	9	1.47 (1.01-2.13)	*0.78*†	21.6 (14.1-33.1)	*0.53*†	18.9 (12.5-28.7)	*0.23*†
CYP1A2 *1F							
AA	100	1.76 (1.59-1.96)		22.1 (19.4-25.2)		12.9 (11.5-14.5)	
AC	70	1.32 (1.17-1.50)	***0.003****	22.0 (18.7-25.8)	*0.63**	16.7 (14.5-19.3)	***0.007****
CC	10	1.71 (1.23-2.39)	***0.02***†	17.8 (11.8-27.1)	*0.52*†	10.3 (7.1-15.0)	*0.25*†
AC + CC	80	1.37 (1.22-1.54)	***0.002***§	21.4 (18.4-24.8)	*0.73*§	15.7 (13.7-17.9)	***0.03***§
CYP1B1							
Val/Val	79	1.54 (1.36-1.74)		23.0 (19.8-26.8)		15.1 (13.1-17.3)	
Val/Leu	83	1.60 (1.42-1.80)	*0.87**	20.9 (18.1-24.2)	*0.64**	13.4 (11.8-15.3)	*0.39**
Leu/Leu	18	1.64 (1.26-2.13)	*0.61*†	20.5 (14.9-28.3)	*0.37*†	12.7 (9.5-17.0)	*0.18*†
CYP17							
TT	66	1.51 (1.32-1.72)		20.5 (17.4-24.0)		14.0 (12.1-16.3)	
TC	89	1.76 (1.49-1.88)	*0.37**	22.9 (19.8-26.4)	*0.59**	13.8 (12.1-15.7)	*0.80**
CC	25	1.45 (1.17-1.79)	*0.88*†	21.8 (16.8-28.3)	*0.48*†	15.1 (11.9-19.2)	*0.73*†

**p* global test for comparison of means; $^{\dagger}p$ for gene dosage effect; $^{\ddagger}p$ for testing a recessive genetic model; $^{\S}p$ for testing a dominant genetic model.